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Suillus flavidus and its ectomycorrhizae with *Pinus wallichiana* in Pakistan

S. Sarwar*, A.N. Khalid, M. Hanif & A.R. Niazi

Department of Botany, University of the Punjab, Quaid-e-Azam Campus, Lahore, 54590, Pakistan
* Correspondence to: *samina_boletus@yahoo.com*

Abstract — *Suillus flavidus* (*Boletales, Suillaceae*) was found associated with *Pinus wallichiana* during a survey of macrofungi from moist coniferous forests of Pakistan. Both the fruiting body and ectomycorrhizae were characterized morpho-anatomically as well as by molecular analysis. This fungus is a new record for Pakistan and its ectomycorrhizae with *Pinus wallichiana* are described for the first time by molecular analysis.

Key words —boletes, ITS, mantle, PCR, rDNA

Introduction

Coniferous forests of Pakistan are located at an elevation of 1373 to 3050 m a.s.l. and are characterized by luxuriant growth of trees such as *Abies pindrow*, *Cedrus deodara*, *Picea smithiana*, *Pinus roxburghii*, *P. wallichiana*, and *Taxus wallichiana*. Among these conifers, some deciduous trees and shrubs of different species also occur (Hussain 1995). Another important feature of these forests is the high level of rainfall during summer (July–August). High rainfall and temperature make an environment suitable for the growth of mushrooms. Most of these fungi form mutualistic symbiotic associations with forest trees in the form of ectomycorrhizae that facilitate tree growth through enhanced nutrient absorption and protection of roots from root pathogens (Marx 1991).

Suillus Gray, a genus with approximately 50 species (Kirk et al. 2008), is characterized by growth under conifers, slimy caps, glandular dots on the stipe, large pore openings that are often arranged radially, and a partial veil that leaves a ring around the stipe or tissue hanging from the cap margin (Kuo 2004). Nine *Suillus* species have been reported from Pakistan (Ahmad et al. 1997; Razaq 2007; Niazi 2008) associated with different conifers.

During field surveys of Pakistan forests, we collected *Suillus* mushrooms under pine trees in order to explore some new and noteworthy species of this

genus. The aim of the present work is to describe the morpho-anatomical features of the *Suillus* collections and their ectomycorrhizae with *Pinus wallichiana* as well as to confirm their identification by molecular analysis.

Materials & methods

The sampling was carried out in coniferous forests of Pakistan located at an elevation of around 2200 m during the 2010 rainy season (July–August). The sporocarps and blocks of soil exactly beneath the fruiting bodies were taken from the rhizosphere of *Pinus wallichiana*. The sporocarps were air dried while the soil blocks containing ectomycorrhizae (ECM) were wrapped in polythene bags and brought to the lab for further analysis. Field data on basidiocarps (site, habitat, association etc) was noted. Specimens were studied macroscopically and microscopically in the laboratory following the methods of Bessette et al. (2000). In the spore dimensions, the first values present the range of lengths and widths, and the values in parentheses present mean spore lengths and widths ± standard deviations followed Q_m ± standard deviation, where Q_m is the mean of Q (= length/width ratio of an individual spore). Other measurements are given as a range with exceptional values in parentheses. ECM were carefully placed in water to clean off soil particles and characterized morphologically under the stereomicroscope. The microscopic description of the ECM follows the terminology of Agerer (1991, 1999). Mantle views, emanating elements, and illustrations were documented with the help of

Table 1: rDNA sequences of *Suillus* species from Pakistan retrieved from GenBank for phylogenetic analysis.

Name	Accession no.	Isolate No.	Origin	Source
Suillus bellinii	AY898621.1	CCMA-22	Spain	Fruitbody
Suillus bovinus	AY898623.1	CCMA-67	Spain	Fruitbody
Suillus brevipes	FJ845440.1	SMI330	Canada	Fruitbody
Suillus caerulescens	EU486453.1	UBC F16304	Canada	Fruitbody
Suillus collinitus	AY953421.1	ECM 14	Spain	Ectomycorrhiza
	DQ440569.1	CCMA-73	Spain	Fruitbody
Suillus flavidus	—	2C	Pakistan	Fruitbodies
	—	2D	Pakistan	Fruitbodies
	—	2H	Pakistan	Fruitbodies
	—	3H	Pakistan	Fruitbodies
	—	H10	Pakistan	Fruitbodies
	—	10B	Pakistan	Fruitbodies
	—	2H 10H	Pakistan	Ectomycorrhiza
	—	3A 11G	Pakistan	Ectomycorrhiza
	—	8G	Pakistan	Ectomycorrhiza
	AJ971403.1	p12	UK	Ectomycorrhiza
	FJ845439.1	SMI206	Canada	Fruitbody
Suillus granulatus	AY898617.1	CCMA-02	Spain	Fruitbody
Suillus grevillei	AF347102.1	EL 38-99	Sweden	Fruitbody
Suillus lakei	DQ367917.1	OUC97024	Canada	Fruitbody
Suillus luteus	DQ440568.1	CCMA-37	Spain	Fruitbody
Suillus mediterraneensis	AY935512.1	CCMA-26	Spain	Fruitbody
Suillus quiescens	GQ249402.1	UC1860306	USA	Fruitbody
Suillus variegatus	AM086444.1	P17 F	UK	Ectomycorrhiza
	AM086446.1	P22 F	UK	Ectomycorrhiza

a camera lucida. Voucher specimens were deposited in the Herbarium, Department of Botany, University of the Punjab, Lahore, Pakistan (LAH).

Molecular analysis

DNA was extracted from dried basidiocarps using Extract N. Amp.TM Plant kit (SIGMA), and the nrDNA was amplified using fungal specific primers pair ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS6-R (5′-TTCCCGCTTCACTCGCAGT-3′). Amplification parameters were denaturation at 94°C for 4 min., then 35 cycles of 45 s at 94°C, 45 s at 54°C, and 1 min 30 s at 72°C, and a final extension at 72°C for 2 min. The purified PCR products were sequenced bidirectionally by Macrogen (South Korea). The sequence was BLAST searched in GenBank for comparison with available sequences. To calculate percent identity, similarity, and divergence, selected sequences were aligned using Clustal W and corrected manually. All ambiguous insertions and deletions were removed prior to further analyses. Percent Identities (PID) and DNA divergence were calculated by DNAStar. Maximum Likelihood and Maximum Parsimony criteria were used to describe the phylogenetic placement with other species included in the present study. Phylograms were made using Mega5.

Taxonomy

Suillus flavidus (Fr.) J. Presl, Wšobecný rostl. 2: 1917 (1846) PLATE 1

PILEUS 3–9 cm wide, convex to hemispherical to nearly plane, sometimes slightly umbonate at maturity, sometimes margins straight and flaring to slightly deflexed with whitish remnants of veil, surface viscid to glutinous when wet, glabrous, yellow to yellowish brown. CONTEXT light yellow, browning when bruised, not bluing. STIPE 3–10 cm long, 1.5–2 cm thick, nearly equal, cylindrical, centric and curved, solid, slightly dry, reddish when young, yellow to white with reddish tinge when mature, whitish glandular dots in some case, whitish thick band like ring present above centre of stipe, color above ring yellow. PORE SURFACE yellow becomes slightly brown upon bruising, adnate and horizontal, pores angular to irregular, infrequent, about 2 per mm, tubes 3–9 mm deep. BASIDIOSPORES ellipsoid to fusoid, smooth, yellow, 9–13 × 4–6 µm, (11.3 ± 1.2 × 5.2 ± 0.6; Q_m= 2.26 ± 0.17). BASIDIA cylindric to long clavate, thick walled, yellowish brown contents visible in Meltzer's, 1–4 sterigmate, 22–26 × 8–10 µm. CYSTIDIA cylindrical to fusoid-ventricose, brown contents visible, thick walled, dark brown, 32–34 × 9–10 µm. PILEIPELLIS long, cylindrical to slightly clavate, thick walled, brown, 77–84 × 18–20 µm, most terminal cells cylindrical to clavate, in clusters and separate also, some are globose from above, dark brown, thick walled, 71–77 × 8–10(–14) µm. CHEMICAL REACTIONS pileipellis reddish in KOH, spores brownish in Meltzer's, light yellow to honey yellow in lactic acid. SMELL & TASTE not distinctive. EDIBILITY edible.

MATERIAL EXAMINED: **PAKISTAN: KHYBER PAKHTUNKHWA,** Ayubia, 2250 m a.s.l., under *Pinus wallichiana* A.B. Jacks., solitary, on ground, 19 July 2010, S. Sarwar (LAH S.B.#6A).

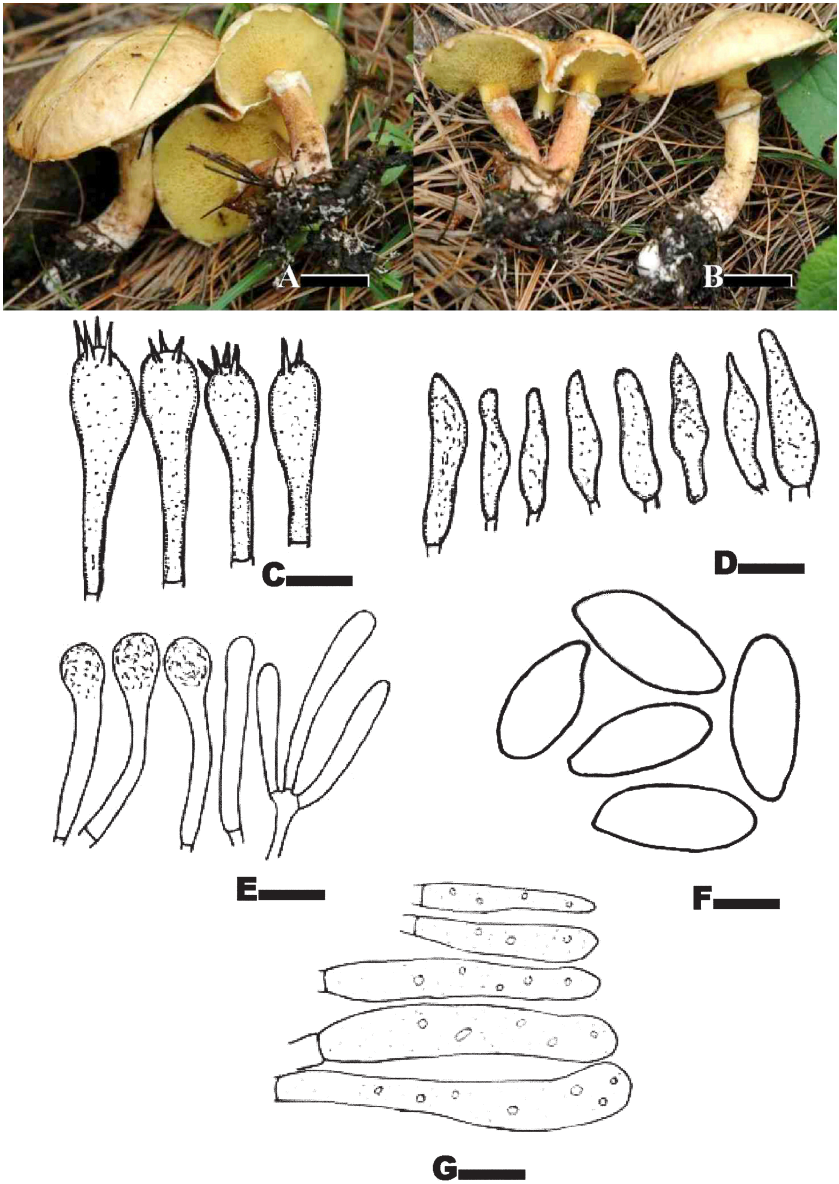

Plate 1. *Suillus flavidus*. A, B, Sporocarps; C, Basidia; D, Cystidia; E, Terminal cells of Pileipellis; F, Basidiospores; G, Pileipellis hyphae. Scale bars: A, B = 2.5 cm; C = 8 µm; D = 11 µm; E = 25 µm; F = 6 µm; G = 16 µm.

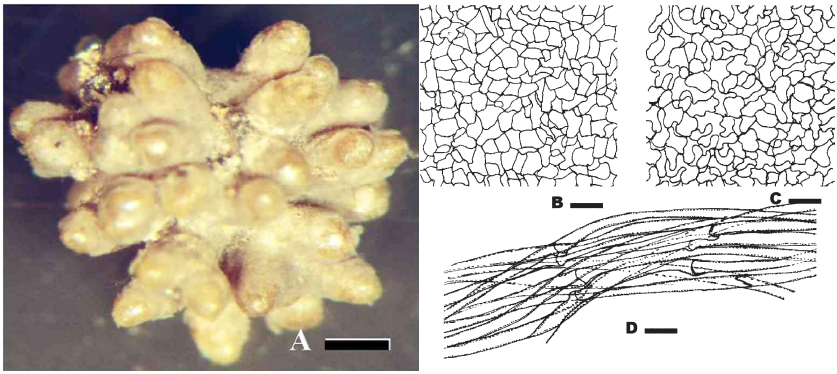

Plate 2. ECM of *Suillus flavidus* with *Pinus wallichiana* and its anatomical features. A, Habit; B, Parenchymatous outer mantle; C, Parenchymatous inner mantle; D, Rhizomorph. Scale bars: A = 1 cm; B = 9 µm; C = 8.5 µm; D = 19 µm.

Description of ectomycorrhizae of *Suillus flavidus*

Morphology **Plate 2a**

Mycorrhizal system found under the fruiting bodies, highly dichotomously branched, system ≤ 5 mm long, with ≤ 0.8 mm thick main axis, unramified ends straight, ≤ 2 mm long, ≤ 0.5 mm diam., color of system reddish brown to dull yellow, older tips dark brown, apices light brown to honey brown, host tissue visible under the sheath. Rhizomorphs frequent and attached at restricted points, whitish brown.

Anatomy of mantle **Plate 2b,c**

Mantle parenchymatous in all layers. Outer mantle parenchymatous (type L, Agerer 1987–2002); cells angular, 5 × 5 µm, honey brown, no matrix material, no septa and clamps observed. Inner mantle parenchymatous, (type M, Agerer 1987–2002); cells slightly angular, 4 × 4 µm, yellowish brown, no matrix material, no septa and clamps.

Anatomy of emanating elements **Plate 2d**

Rhizomorphs slightly differentiated (type A, Agerer 1991), cells 26 × 7 µm, light brown, clamps common, septa less common and only at clamps, H-shaped anastomoses without clamps present. Emanating hyphae absent.

Material examined: **PAKISTAN: Khyber Pakhtunkhwa,** Ayubia, 2250 m a.s.l., Ectomycorrhizae Under *Suillus flavidus* near rhizospere of *Pinus wallichiana*, 19 July 2010, S. Sarwar (LAH S.B.#6B).

Molecular identification and phylogeny

When ITS-rDNA sequence was submitted for similarity with GenBank data, it was identified as *Suillus flavidus* with 98% maximum identity and 100%

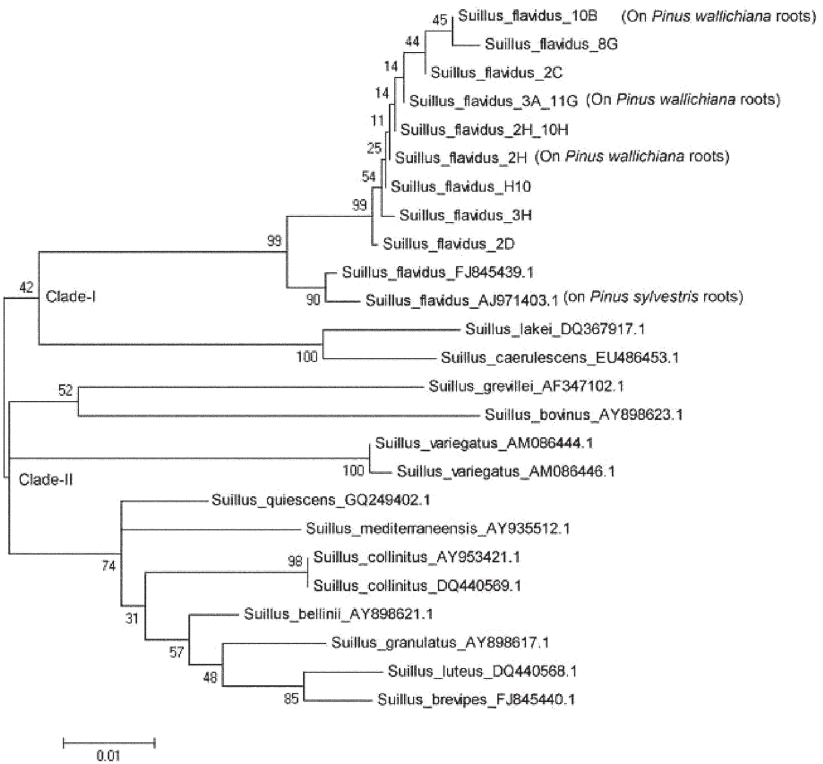

Figure 1: Molecular phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Poisson correction model (Zuckerkandl & Pauling 1965). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically as follows: when the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 25 sequences. There were a total of 426 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011).

query coverage with *S. flavidus* (FJ845439.1) from Canada. The phylogenetic analysis includes 25 sequences from 13 species (Table 1). For phylogenetic analysis, 426 genetic characters were used in aligned datasheet containing 330 conserved sites, 91 variable sites, and 66 parsimony sites. Phylogenetic analysis also supports the morpho-anatomic and molecular identification. The phylogram based on maximum likelihood criterion is represented by two major clades (Fig. 1). Clade I comprises 11 sequences from *S. flavidus*, *S. lakei* (Murrill) A.H. Sm. & Thiers (DQ367912.1), *S. caerulescens* A.H. Sm. & Thiers

(EU486453.1), and *S. grevillei* (Klotzsch) Singer (AF347102.1); their clustering is not highly resolved (bootstrap = 42%). *Suillus flavidus,* which occupies the top position in the phylogram, is represented by nine Pakistan sequences (six from fruitbodies and three from *P. wallichiana* ectomycorrhizal roots) and two GenBank sequences. The Pakistan rDNA-ITS sequences shared 100% genetic similarity with one another and about 98% with *S. flavidus* FJ845439.1. The two neighboring *S. flavidus* sister clades in clade 1 have a different topological placement due to intraspecific variations within the region sequenced. This species has a 92.1% genetic similarity with *S. lakei* DQ367912.1 and 91.6% with *S. caerulescens* EU486453.1. Genetic divergence was also measured for *S. flavidus* with all the sequences included in the analysis. No genetic divergence was found among the rDNA-ITS of *S. flavidus* from Pakistan (Fig. 2), and there was little genetic divergence (0.5–2.5) compared with *S. flavidus* FJ845439.1.

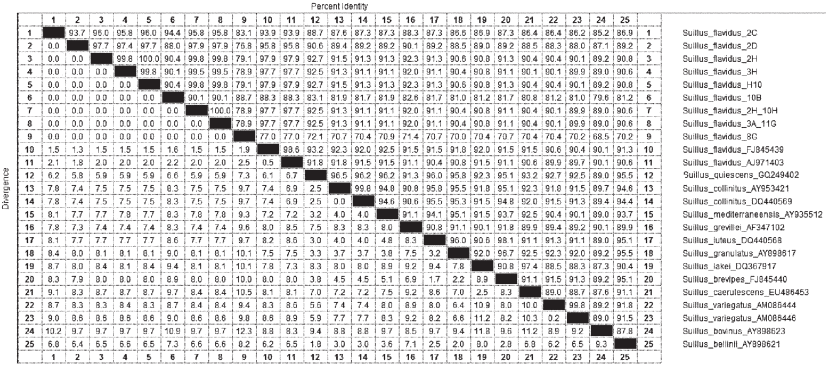

Figure 2: Percent divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by MegAlign (DNASTAR). Percent similarity compares sequences directly without accounting for phylogenetic relationships.

Discussion

We report *S. flavidus* and its ectomycorrhizae with *P. wallichiana* for the first time from Pakistan. This fungus is characterized by a convex to hemispheric yellowish pileus with reddish brown spots on the margin and typically small hanging veil remnants and a stipe with a prominent ring. Spores are smooth and range from light to dark brown. *Suillus flavidus* resembles *S. lakei*, which differs in a pileus surface covered with dull reddish brown scruffies and a stipe with reddish streaks and without glandular dots. The similar *S. caerulescens* also lacks glandular dots but has occasional appressed hairs on the stipe. Finally, *S. grevillei,* which is also resembles *S. flavidus,* has a somewhat reticulated stipe that lacks the glandular dots that characterize *S. flavidus* (Bessette et al. 2000).

We characterize *S. flavidus–P. wallichiana* ectomycorrhizae here for the first time by noting their highly dichotomously branched ramifications with parenchymatous inner and outer mantle surfaces, slightly differentiated rhizomorphs, and no emanating hyphae. Different Pakistani ectomycorrhizae associated with *Cedrus deodara* have recently been molecularly analyzed (Hanif et al. 2010) but there is no literature on molecular description of ectomycorrhizae of boletes from Pakistan. We report *S. flavidus* as new record for Pakistan and believe that *P. wallichiana* is documented here as a new ectomycorrhizal host for *S. flavidus* for the first time.

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